Kidney International, Vol. 55 (1999), pp. 1518–1527

Effects of FK506 in rat and human resistance arteries

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Effects of FK506 in rat and human resistance arteries.

Background. FK506 is widely used in organ transplantation and causes hypertension. However, little is known about the impact of the drug on the cardiovascular system.

Methods. We therefore investigated the effect of FK506 on resistance artery and blood pressure responsiveness to vasoconstrictors and vasodilators. Studies were conducted *in vitro* using human and murine resistance artery, *ex vivo* in resistance artery isolated from rats treated with FK506 (6 mg/kg/day), and *in vivo* in conscious, treated animals.

Results. In vitro exposure (24 hr) of human and rat resistance artery to FK506 (1000 ng/ml) increased the sensitivity to norepinephrine (NE) and impaired the response to acetylcholine (Ach) and sodium nitroprusside (SNp). In contrast, arteries isolated from rats given FK506 for eight days showed a reduced sensitivity to NE ($P < 0.05$) and a normal endothelium-dependent relaxation. Their incubation with L-arginine caused a significant reduction in Ach sensitivity in the FK506 group ($P < 0.05$) but not in controls, suggesting enhancement of nitric oxide production by the drug. The sensitivity to SNp was reduced, as in the *in vitro* experiments ($P < 0.05$). Rats given FK506 for eight days presented blood pressure similar to that in controls but also presented signs of a compensatory response to excess vasodilation: tachycardia ($P < 0.01$), reduced blood pressure sensitivity to NE and Ach, blunted heart rate response to both agonists, and exaggerated hypotension at high doses of Ach. After 21 days of treatment, blood pressure remained similar to that in controls, but resistance artery showed further functional deterioration, with significant impairment of the maximum responses to Ach and to SNp.

Conclusion. FK506 presents significant vascular toxicity affecting mainly smooth muscle relaxation and alters vascular hemodynamics. The data suggest that similar cardiovascular changes may occur in transplant patients and represent the forerunner of hypertension often seen with more prolonged use of the drug.

The immunosuppressant agent FK506 (tacrolimus) is a macrolide antibiotic of fungal origin that selectively inhibits T-lymphocyte activation. Like cyclosporine, another potent immunosuppressant, FK506, interferes with intracellular signaling mechanisms in a calcium-dependent manner, resulting in the inhibition of the transcription of genes regulating the syntheses of cytokines [1]. The adverse effects of FK506 are similar to those related to cyclosporine's use and include hyperglycemia, dyslipidemia, nephrotoxicity, neurotoxicity, and hypertension [2, 3]. However, differences in the intensity and incidence of these side effects may exist. For example, hypertension, which is an important side effect of both drugs, in some investigations has been found to be less severe and frequent in patients receiving FK506 [4–8]. In their study, Canzanello et al observed that hypertension occurred in 82% of their liver transplant patients treated with cyclosporine and in only 33% of those treated with FK506 [9]. Spencer, Goa and Gillis in their recent review, reached a similar conclusion in which the frequency of adverse effects of both immunosuppressive drugs was compared [10]. In particular, the authors reported that although FK506 was associated with a higher incidence of diabetes mellitus, neurotoxicity, and nephrotoxicity, cyclosporine caused more hyperlipidemia, hypertension, hirsutism, and gingival hyperplasia in patients submitted to different modalities of organ transplantation.

Intense research has been devoted to the elucidation of the pathogenesis of cyclosporine-induced hypertension. Several studies have now established that the drug increases systemic [11], renal [8], and coronary [12] vascular resistance. Others have shown that increased vascular resistance is a consequence of drug-induced impairment of arterial relaxation. Our own work showed, in particular, that the resistance vessels of rats turned hypertensive by chronic cyclosporine treatment and presented a pronounced decrease in endothelium-dependent and -independent relaxation associated with reduction in the sensitivity to vasoconstrictors [13].

Comparatively, there is little information on the vascular effects of FK506, and the mechanism responsible for the hypertensive effect of the drug remains poorly understood. The drug may increase vascular resistance,

Key words: vascular tone, hypertension, blood pressure, transplantation, vasoconstriction, hemodynamics.

Received for publication April 3, 1998
and in revised form October 19, 1998
Accepted for publication October 19, 1998

although to a lesser degree than cyclosporine [11, 12]. However, to our knowledge, no study has been conducted to clarify the vascular changes associated with FK506 exposure in relation to its hemodynamic effects. Our goal in this work was therefore to investigate the effects of FK506 on blood pressure, blood pressure responsiveness, and vascular function, as defined by responses to vasoconstrictors and vasodilators. Particular emphasis was given to the exploration of the functional alterations in resistance vessels, as small arteries are believed to play a central role in the peripheral control of blood pressure [14]. Most of the experiments were conducted in animals treated with FK506. However, the vascular effects of FK506 were also characterized in animal and human resistance arteries exposed *in vitro* to the drug.

METHODS

Subjects

Small arteries were isolated from fat tissue specimens obtained from seven individuals (3 males and 4 females, age 32 to 78 years) during diagnostic procedures performed under anesthesia. The protocol was approved by the institutional review board. No subject had been exposed to any immunosuppressive drug at the time of surgery. Arterial segments (approximately 200 μm in diameter and 1 to 3 mm in length) were dissected and processed as described later here.

Animals

Twelve- to 14-week-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used for the animal experiments. They were kept on a 12-hour dark–light cycle, with free access to water and to standard rat chow (Ralston-Purina Co., St. Louis, MO, USA). The animals were housed in metabolic cages for urine collection as dictated by the protocols.

Measurement of vascular reactivity in resistance vessels in the rat

Mesenteric artery resistance vessels were isolated and prepared as previously described [15]. Briefly, the second branches of mesenteric arteries were isolated, excised, and rinsed with saline. Arterial segments (2 to 3 mm long) were then mounted in a wire myograph and set at their optimal length for force development by construction of a length-tension curve. The lumen diameter and the wall thickness of the arteries were measured first, using a filar micrometer eyepiece. The reactivity to vasoconstrictors and vasodilators was determined next after "wake-up" stimulation and a 30-minute equilibration period in a physiological buffer [16]. Responses were reported as absolute force normalized to axial length of the vessel (active tension in mN/mm) for contraction or percentage of precontraction with norepinephrine (NE) for relaxation (100% equals no relaxation). The sensitivity to agonists was calculated using computerized nonlinear curve fitting and expressed as the efficient dose inducing 50% of the maximum response (ED_{50}).

Experimental protocols

Four experimental protocols were performed. Protocol 1 aimed to characterize the changes in vascular reactivity induced by *in vitro* exposure to FK506. Protocols 2 through 4 evaluated the effect of chronic short-term and long-term administration of FK506 in rats on blood pressure and on vascular function.

In protocol 1, human and animal (Sprague-Dawley rats) arteries were incubated for 24 hours with either 1000 ng/ml FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) or vehicle (ethanol, 0.1%, vol/vol). Incubation was performed at 37°C under a 95% O_2 and 5% CO_2 atmosphere and in Dulbecco's modified essential medium (DMEM) supplemented with antibiotics (penicillin and streptomycin) and with insulin and transferrin [16]. Two arterial segments per animal or per fat biopsy were prepared and studied simultaneously after incubation with either FK506 or vehicle. The following agonists were assayed: NE, acetylcholine (Ach), and sodium nitroprusside (SNp).

In protocol 2, we studied the effects of short-term (eight days) treatment with FK506 on arterial blood pressure and vascular function in rats. Two groups of animals ($N = 11$ per group) received either FK506, administered at the dose of 6 mg/kg/day, by gavage, once a day, or the vehicle for oral formulation (Fujisawa Pharmaceutical). Body weight was recorded daily. Systolic blood pressures were measured daily at 9:00 a.m. using the tail-cuff method (Narco-Bio, Houston, TX, USA). Animals were accustomed to blood pressure measurements for one week (baseline) prior the treatment period. For estimation of the effect of FK506 on body weight and blood pressure, the measurements corresponding to the last three days of the baseline period were averaged and compared with the average weight or blood pressure corresponding to the last three days of the treatment period. The animals were sacrificed in the morning after a 16- to 18-hour fast. Blood samples were collected for the determination of the plasma levels of cholesterol, urea, creatinine, calcium, and magnesium. Animals were then killed by decapitation under ether anesthesia, and the vascular reactivity of the mesenteric resistance vessels was studied. The following drugs were assayed in sequence: NE, Ach, and SNp. The dose response to Ach was established first in basal conditions and then after a 20-minute incubation with 300 μM L-arginine. In a parallel experiment (6 animals per group), we determined glomerular filtration rate (GFR) using ^{14}C-inulin

[17] at the eighth day of the experiment and measured trough (24-hr postgavage) plasma levels of FK506.

Protocol 3 evaluated the effect of short-term (8 days) treatment with FK506 (6 mg/kg/day) on blood pressure responsiveness in the rat ($N = 10$ animals). The results were compared with those observed in control rats receiving the vehicle of the drug. The animals were studied conscious, one day after femoral and jugular catheters were implanted. Basal heart rate and mean arterial pressure (MAP) were determined after a 30-minute infusion of saline using a direct blood pressure measurement system (Kent Scientifics Corp., Litchfield, CT, USA). Blood pressure responsiveness to NE was next determined using bolus injections of NE (0 to 5 μg/kg body wt) in the jugular vein. Blood pressure and heart rate were allowed to return to baseline between each injection (approximately 5 min). After the last dose of NE, saline was infused for 20 minutes until the responsiveness to Ach was studied. For this, incremental doses of Ach (0 to 10 μg/min/300 g body wt) were infused until steady depression of MAP was observed (approximately 5 min). Each Ach infusion was followed by a 10-minute infusion of saline that allowed the return to baseline of both heart rate and blood pressure. Dose–response curves were established using the peak response for NE or the plateau response for Ach.

Protocol 4 consisted in repeating the experiments of protocol 2 after 21 days of treatment with FK506 (6 mg/kg/day, long term). The animals were housed in metabolic cages for the last 10 days before sacrifice. In the vessel experiments, incubation with L-arginine was not performed. Instead, the Ach dose response was established first in basal conditions, after incubation with 10^{-5} M indomethacin for 20 minutes and then in the presence of both indomethacin and 1 μM methylene blue. The response to SNp was studied last. Sodium balance was calculated using food consumption and urinary sodium excretion determined on samples collected during the last six days of the treatment period. Blood samples were obtained for the determination of the plasma levels of urea, creatinine, total cholesterol, calcium, magnesium, and trough blood levels of FK506.

Analytical methods

Urinary sodium was measured by ion selective electrode (E4A; Beckman Instruments Inc., Palo Alto, CA, USA). Plasma creatinine, urea, total cholesterol, calcium, and magnesium were measured by spectrophotometry using a centrifugal analyzer (Cobas Bio; Roche Diagnostica, Nutley, NJ, USA). Plasma FK506 levels were determined using double-sandwich enzyme-linked immunosorbent assay (ELISA; Fujisawa Pharmaceutical).

Statistical analysis

Values are expressed as mean ± SEM. Experimental groups were compared using analysis of variance, paired and unpaired *t*-tests, or nonparametric tests (Mann–Whitney and Wilcoxon's tests) when appropriate.

RESULTS

Protocol 1

In these experiments, we characterized the direct effect of FK506 on resistance arteries using vessels incubated *in vitro* with the drug. Initial experiments were conducted with human resistance arteries exposed for 24 hours to 100 ng/ml FK506. This concentration, which is close to whole-blood peak concentrations achieved after oral administration in transplant patients [10], proved to have no effect on the reactivity to NE, Ach, and SNp (data not shown). We thus decided to expose the arteries to pharmacological concentrations of FK506 (1000 ng/ml, that is, approximately 10 to 20 times higher than therapeutic concentrations). This approach had been used successfully in previous studies to characterize the direct effect of cyclosporine A on vascular function [13].

The wall-to-lumen ratio of human vessels exposed to 1000 ng/ml FK506 for 24 hours did not differ from controls (0.094 ± 0.007 vs. 0.101 ± 0.008, for FK506 and control, respectively, P = NS). Human arteries exposed *in vitro* to FK506 showed an increased sensitivity to NE [ED_{50} was 267 ± 47 (FK506) and 514 ± 146 nM (control, $P < 0.01$)]; however, the maximum response to NE was not affected: 2.23 ± 0.45 and 2.21 ± 0.35 mN/mm for FK506 and control (P = NS). The maximum response to Ach was slightly but significantly decreased in the FK506 vessels by comparison to controls (Fig. 1A). The sensitivity to Ach was not affected: 37 ± 10 nM and 79 ± 40 nM for control and FK506, respectively (P = NS). The sensitivity to SNp was significantly reduced, as indicated by the shift to the right of the SNp dose–response curve (Fig. 1B). The maximum response to SNp was also impaired, although modestly (same figure).

Similar results were obtained with rat mesenteric arteries incubated for 24 hours with 1000 ng/ml FK506. There was no significant effect of the drug on the wall-to-lumen ratio of the arteries (not shown) and on the maximum response to NE (4.27 ± 0.30 vs. 3.98 ± 0.22 mN/mm for FK506 and control, respectively, P = NS). However, as observed with human vessels, the sensitivity to NE was increased by FK506: ED_{50} was 194 ± 35 nM (FK506) and 296 ± 54 nM (controls, $P = 0.015$). The maximum response to Ach was minimally but significantly impaired (4.9 ± 1.1 vs. 0.8 ± 0.7% of precontraction for FK506 and controls, respectively, $P < 0.02$), and the sensitivity was reduced: Ach-ED_{50} was 88 ± 20 nM (FK506) and 67 ± 21 (controls, $P < 0.05$). Finally, the SNp sensitivity was reduced [ED_{50} were 230 ± 76 nM and 109 ± 44 nM for FK506 and control, respectively

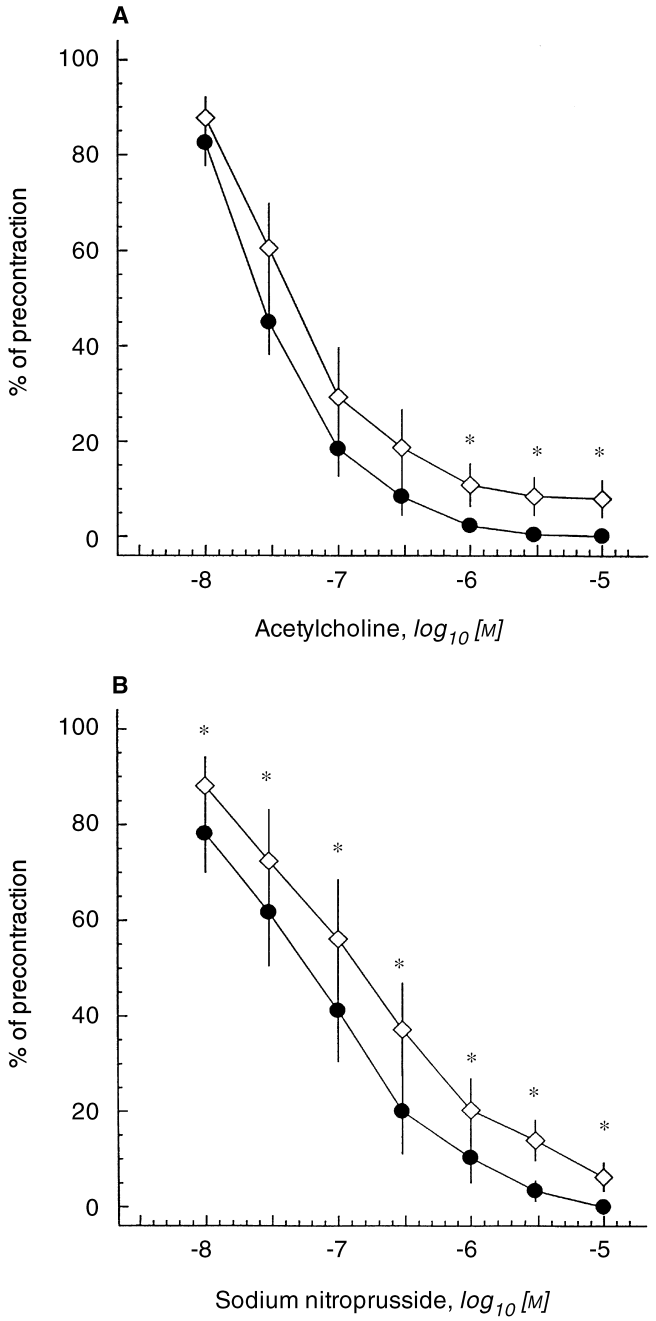

Fig. 1. ***In vitro*** **effect of FK506 on human arteries (*N* = 7).** Isolated arteries were exposed for 24 hours with 1000 ng/ml FK506 (◇) or vehicle (●), contracted with norepinephrine (NE), and challenged with cumulative dose of acetylcholine (*A*) or sodium nitroprusside (*B*), as described in the **Methods** section. Asterisks indicate a significant ($P < 0.05$) difference between the two groups. SNp-ED_{50} values in controls was 158 ± 97 nM and in FK506 was 264 ± 123 nM ($P < 0.01$).

($P < 0.01$)]; however, no significant change in the maximum relaxation to SNp was observed (not shown).

Protocol 2

Body weight was similar in both control and FK506 groups at baseline (316.1 ± 5.3 vs. 313.5 ± 7.9 g, P = NS). After eight days of treatment, the body weight of the animals receiving vehicle increased but did not change significantly in the rats treated with FK506: body weight gain was 19.8 ± 3.3 and −3.5 ± 3.1 g ($P < 0.001$) for controls and FK506 animals. Baseline systolic blood pressure also did not differ between the groups (130.7 ± 4.4 vs. 132.6 ± 3.4 mm Hg, P = NS). Post-treatment systolic blood pressure was 132.2 ± 3.7 and 137.1 ± 3.1 mm Hg for vehicle and FK506, respectively. The difference between pretreatment and post-treatment blood pressure in the two groups was not significant: 4.50 ± 2.6 mm Hg vs. 1.50 ± 2.5 (P = NS).

Table 1. Effect of short-term (8 days) FK506 treatment on the plasma levels of creatinine, urea, total cholesterol, calcium and magnesium, and on glomerular filtration rate (GFR) (N = 11 animals per group)

Variable	Control	FK506	P
Creatinine *μM*	64 ± 4	54 ± 3	NS
Urea *mM*	6.4 ± 0.4	8.0 ± 0.3	< 0.001
Total cholesterol *mM*	1.52 ± 0.08	1.82 ± 0.11	< 0.05
Calcium *mM*	2.3 ± 0.02	2.29 ± 0.03	NS
Magnesium *mM*	0.55 ± 0.02	0.45 ± 0.03	< 0.01
GFR *ml/min/100 g body wt*	1.1 ± 0.2	1.2 ± 0.1	NS

Table 1 shows the effect of short-term FK506 treatment on blood chemistry and on GFR. Compared with vehicle, FK506 caused a significant increase in the plasma levels of urea and total cholesterol and a reduction in plasma magnesium concentrations. Plasma levels of creatinine and calcium were not affected by FK506. When plasma creatinine levels were corrected by body weight, still no significant difference could be observed: 0.21 ± 0.01 (control) vs. 0.18 ± 0.01 (FK506, P = NS). There was no difference in GFR between the groups. FK506 attained levels considered to be in the low therapeutic trough range, as seen in patients receiving the drug (5.95 ± 0.96 ng/ml).

The arteries from animals treated with FK506 did not show any significant alteration of their wall-to-lumen ratio when compared with control: 0.0823 ± 0.004 vs. 0.0894 ± 0.006 for FK506 and control, respectively (P = NS). However, departing from the results obtained in the human and in the rat *in vitro* studies, resistance vessels of rats treated with FK506 presented a reduced sensitivity to NE, as demonstrated by a shift of the dose–response curve to NE to the right (Fig. 2): NE-ED_{50} was 2276 ± 402 nM (FK506) and 1162 ± 160 nM (control, $P < 0.05$). The maximum response to the highest concentration of NE employed was significantly reduced in the FK506 group (Fig. 2). However, the calculated maximum contraction to NE, taking into account all points of the dose–response curve, was not different from controls: 5.8 ± 0.2 vs. 5.6 ± 0.3 mN/mm for controls and FK506, respectively (P = NS). Maximum relaxation and sensitivity to Ach were not impaired by FK506 in basal condi-

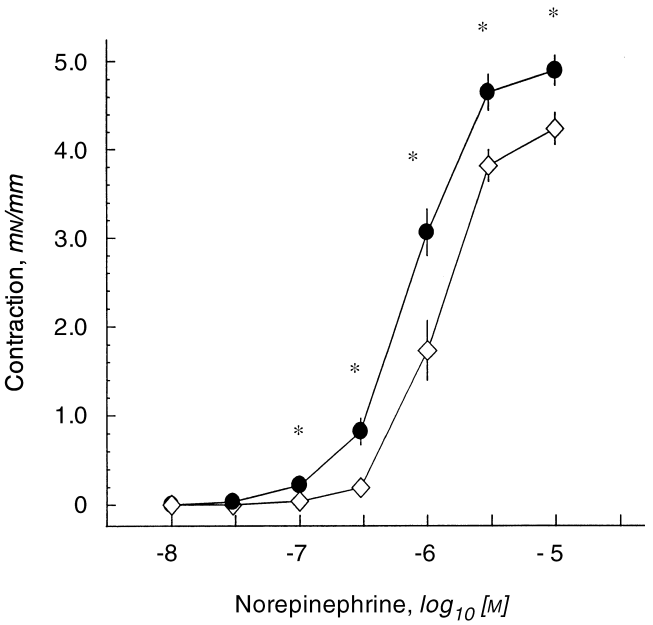

Fig. 2. Effect of FK506 (short-term treatment) on NE-induced contraction in isolated rat arteries (*ex vivo* studies). Arteries isolated from FK506-treated (◇) and control (●) animals ($N = 11$ per group) were challenged with a cumulative dose of NE. Asterisks indicate a significant ($P < 0.05$) difference between the two groups.

tions (Fig. 3A). However, preincubation of the vessels with L-arginine reduced the sensitivity to Ach in the FK506 group: Ach-ED_{50} increased from 142 ± 46 nM (basal) to 290 ± 72 nM (L-arginine, $P < 0.05$). No such change was observed in control arteries exposed to L-arginine: Ach-ED_{50} was 87 ± 20 nM (basal) and 81 ± 20 (L-arginine). Thus, after repletion with L-arginine, FK506 arteries had a reduced sensitivity to Ach as compared with controls (Fig. 3B). The response to SNp was next investigated in order to test the endothelium-independent relaxation. Sensitivity to SNp was significantly reduced with FK506, whereas the maximum relaxation was not affected (Fig. 4).

Protocol 3

After eight days of FK506 administration, MAP directly measured in conscious animals was not different from the controls (154.3 ± 8.6 vs. 148 ± 5.6 mm Hg, P = NS). However, there was a significant increase in heart rate (521 ± 13 vs. 436 ± 13, $P < 0.001$). The evaluation of pressor responsiveness to NE was coherent with the results obtained in the *ex vivo* studies (protocol 2). The MAP dose–response curve was displaced significantly to the right, configuring a reduction in the sensitivity to NE (Fig. 4A). Maximum response to NE was not affected (Fig. 5A). The heart rate response to NE was significantly attenuated in the rats receiving FK506 as compared with controls (Fig. 5B). FK506 caused a reduction in sensitivity to Ach, as evidenced by the reduced fall in MAP following the infusion of low concentrations of Ach (Fig. 6A). In contrast, a greater fall in blood pressure was achieved in FK506 animals with higher concentrations of Ach (same figure). At these Ach concentrations, animals receiving FK506 experienced a blunted heart rate response compared with controls (Fig. 6B).

Protocol 4

After 21 days of treatment, trough levels of FK506 were 3.64 ± 0.05 ng/ml, still within the human therapeutic range. Indirect systolic blood pressure was not different from control: 155.1 ± 4.3 mm Hg vs. 159.1 ± 3.6 mm Hg for FK506 and control, respectively (P = NS). During the same period, animals treated with FK506 developed a positive sodium balance contrary to the control animals (2.11 ± 0.23 mmol/day vs. 0.97 ± 0.08 mmol/day, $P < 0.001$). In spite of that, animals treated with FK506 gained only 6.7 ± 8. g body wt, whereas controls rats gained 36.3 ± 8.4 g during the experiment ($P < 0.02$). As can be seen in Table 2, FK506 caused a significant increase in plasma urea ($P < 0.001$). On the other hand, plasma creatinine was not significantly changed. Plasma magnesium concentration was reduced, and total cholesterol was increased in animals treated with FK506.

The wall-to-lumen ratio of the mesenteric arteries was not significantly changed by 21 days of FK506 treatment. It was 0.108 ± 0.003 for FK506 and 0.098 ± 0.005 for control (P = NS). In agreement with the results of the short-term study, the sensitivity to NE was significantly reduced in the FK506 animals: 1023 ± 85 versus 724 ± 90 nM for FK506 and control, respectively ($P < 0.02$). The maximum response to NE was similar in the two groups: 5.13 ± 0.24 (FK506) and 4.72 ± 0.26 mN/mm (control, P = NS). Contrary to the short-term experiment, the maximum response to Ach was reduced by FK506 (Fig. 7A). However, the sensitivity to Ach was preserved, similar to the results obtained in the short-term protocol: 79 ± 14 and 69 ± 11 nM for control and FK506, respectively (P = NS). Because Ach is known to stimulate the release of thromboxane, a vasoconstrictor prostaglandin, we tested the hypothesis that the reduced maximum-relaxation response was due to interference by that prostanoid. Preincubation of the resistance vessels of FK506 animals with indomethacin, a prostaglandin synthesis inhibitor, did not restore maximum relaxation to normal (Fig. 7B). We then tested the hypothesis that the altered maximum Ach-induced relaxation was the consequence of decreased production of nitric oxide (NO). For this, we used methylene blue (MB), a NO quencher [18]. The rationale for using MB was that vessels with decreased NO production would be less responsive to MB, thus suppressing the difference between control and FK506. As shown in Figure 7B, MB decreased

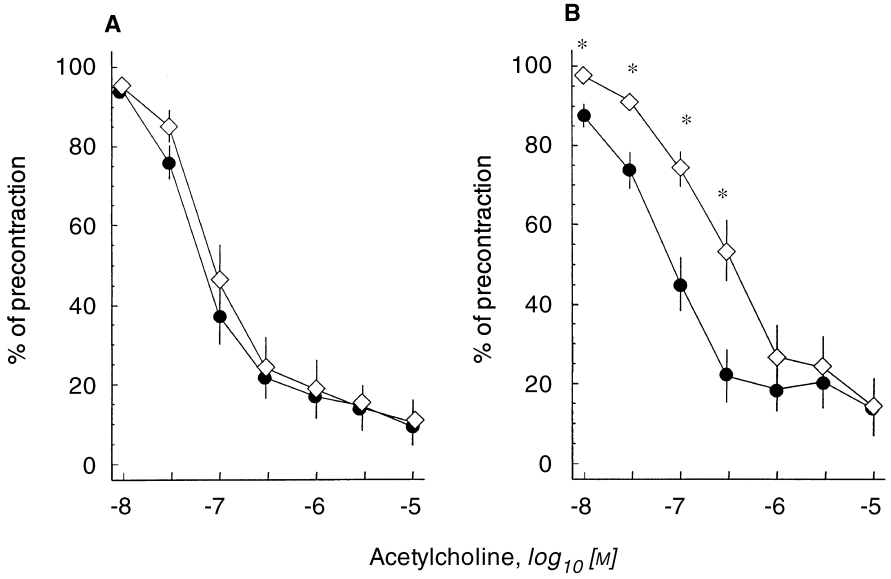

Fig. 3. Effect of FK506 (short-term treatment) on Ach-induced relaxation in isolated rat arteries (*ex vivo* studies). Arteries isolated from FK506-treated (◇) and control (●) animals ($N = 11$ per group) were contracted with NE and challenged with cumulative dose of Ach. Relaxation to Ach was determined in basal conditions (*A*) or after incubation with L-arginine (*B*) (300 μM for 20 min). Asterisks indicate a significant ($P < 0.05$) difference between the two experimental groups.

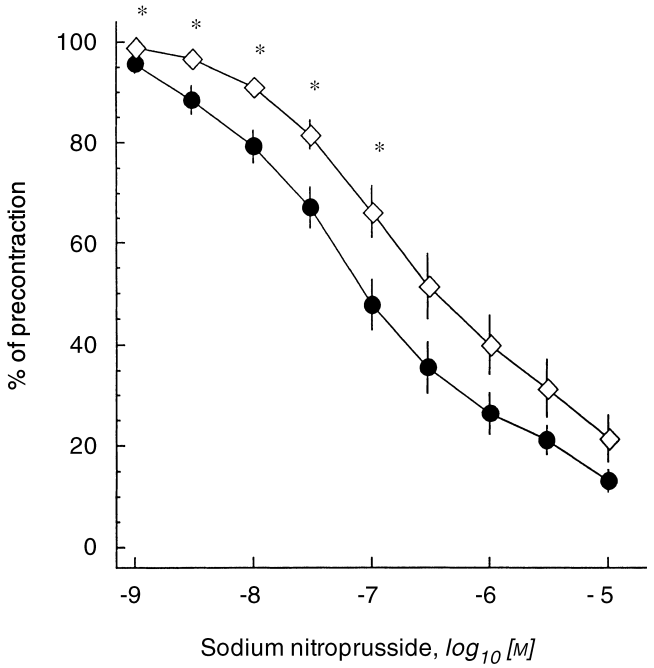

Fig. 4. Effect of FK506 (short-term treatment) on SNp-induced relaxation in isolated rat arteries (*ex vivo* studies). Arteries isolated from FK506-treated (◇) and control (●) animals ($N = 11$ per group) were contracted with NE and challenged with cumulative dose of SNp. Asterisks indicate a significant ($P < 0.05$) difference between the two experimental groups.

the maximum response to Ach in both groups but did not suppress the difference between FK506 and controls. We finally tested the response to exogenous NO using SNp. The results, presented in Figure 8, showed that FK506 impaired both sensitivity and maximum response to SNp.

DISCUSSION

We started this investigation exploring the hitherto not reported direct effect of FK506 on the vasoconstrictor and vasodilator responses of human resistance vessels *in vitro*, complemented with observations in the rat. We considered this approach relevant because it freed us from the interference of confusing factors operating in the whole organism, and because the effects of FK506 on the cardiovascular system might vary in the different species. In this work, human resistance vessels preincubated for 24 hours with FK506 presented an increased sensitivity to NE and moderate but significant alterations in endothelium-dependent and -independent relaxation. Similar alterations were observed in the rat vessels. The increased response to NE may be ascribed to the direct effect of FK506 on neurotransmitter release from adrenergic nerve terminals [19], but more likely is secondary to a decrease in vasodilatory tone, as evidenced by the compromised response to Ach and to SNp. It should be emphasized that the concentrations of FK506 that elicited these effects were well above of the trough levels achieved in patients, and that experiments conducted with concentrations close to those considered clinically adequate did not elicit any change in vascular function. That means that the vascular effects registered *in vitro*, although showing the potential vascular toxicity of FK506, do not necessarily reflect these effects under normal clinical situations. For that reason, we also ran *ex vivo* and *in vivo* experiments in animals because toxicological studies in normal humans should not be acceptable.

We showed that animals treated with FK506 do not develop hypertension, either during the short- or long-term course of the drug. This was found by measuring

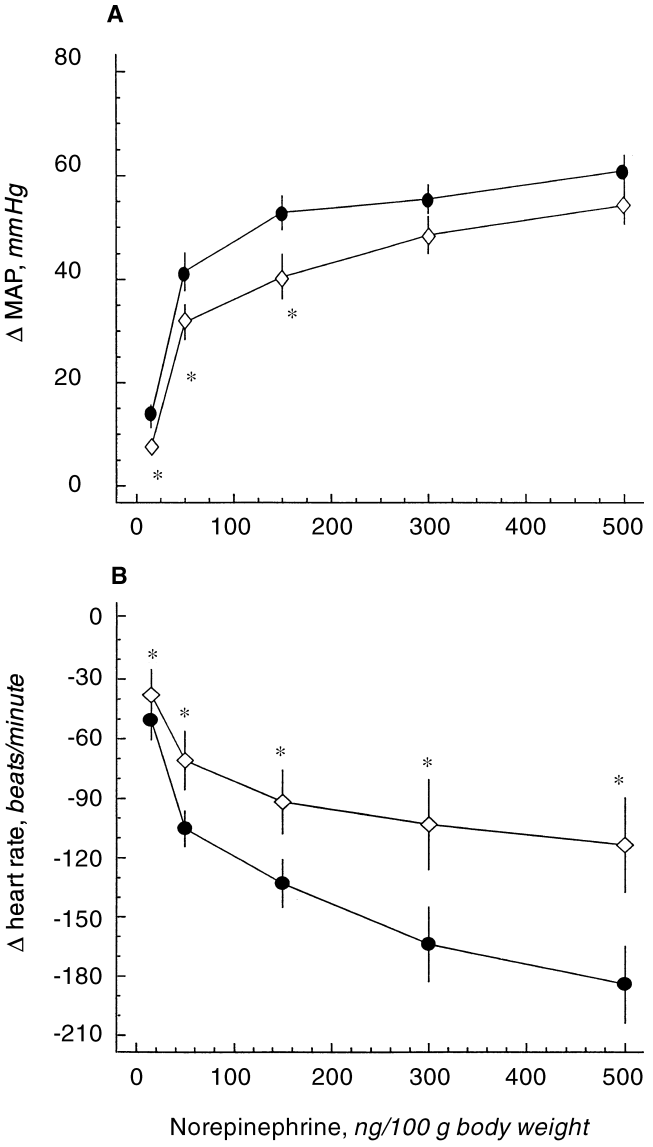

Fig. 5. Effect of FK506 (short-term treatment) on blood pressure and heart rate responsiveness to NE in the rat (*in vivo* studies). FK506-treated (◇) and control (●) animals ($N = 10$/group) were instrumented as described in the **Methods** section. Mean arterial pressure (MAP; *A*) and heart rate (*B*) responsiveness (peak response − baseline) was determined after bolus injections of NE (**Methods** section). Asterisks indicate a significant ($P < 0.05$) difference between the two groups.

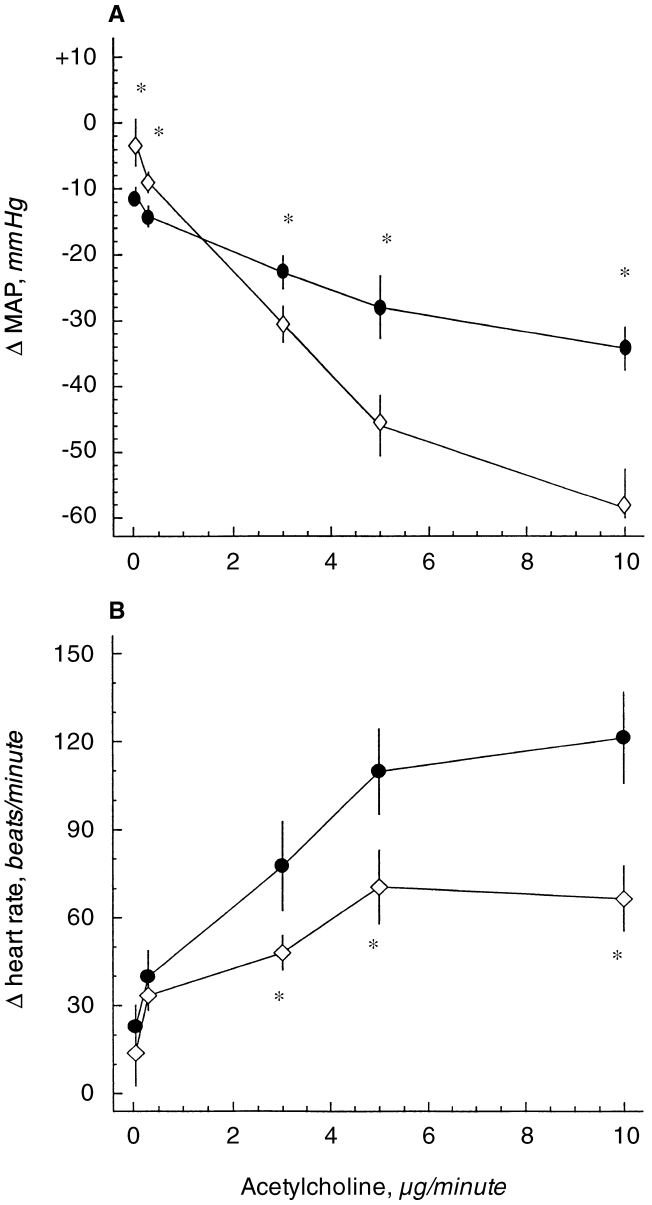

Fig. 6. Effects of FK506 (short-term treatment) on blood pressure and heart rate responsiveness to Ach in the rat (*in vivo* studies). FK506-treated (◇) and control (●) animals ($N = 10$/group) were instrumented as described in the **Methods** section. Blood pressure [mean arterial pressure (MAP), *A*] and heart rate (*B*) responsiveness (peak response − baseline) were determined after a five-minute infusion of Ach (**Methods** section). Asterisks indicate a significant ($P < 0.05$) difference between the two groups.

blood pressure indirectly, as well as by the direct method, in the conscious animal. The absence of a hypertensive effect of FK506 occurred despite concomitant sodium retention during the administration of the drug and with blood levels within human therapeutic range [20]. These concentrations have also been proved to be immunosuppressive in the rat [21]. Significant elevation in blood pressure with FK506 can, however, be achieved in the rat with higher, intravenous doses of the drug [19]. These findings contrast with the observations that moderate doses of cyclosporine in the same model are usually

Table 2. Effect of long-term (21 days) FK506 treatment on the plasma levels of creatinine, urea, total cholesterol, calcium and magnesium ($N = 9$ animals per group)

Variable	Control	FK506	*P*
Creatinine *mM*	67 ± 4	68 ± 5	NS
Urea *mM*	5.3 ± 0.3	8.4 ± 0.6	< 0.001
Total cholesterol *mM*	1.51 ± 0.08	2.44 ± 0.17	< 0.001
Calcium *mM*	2.48 ± 0.02	2.57 ± 0.02	NS
Magnesium *mM*	0.70 ± 0.02	0.52 ± 0.02	< 0.01

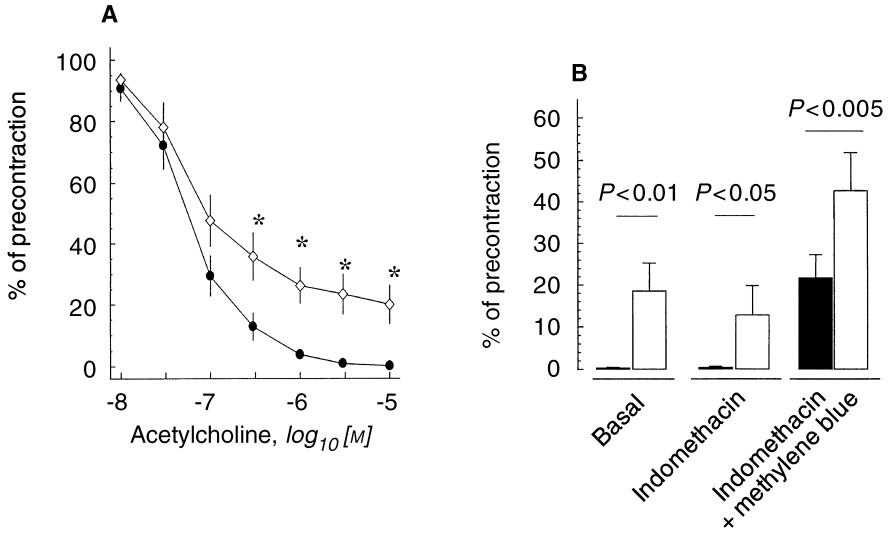

Fig. 7. Effect of FK506 (long-term treatment) on Ach-induced relaxation in isolated rat arteries (*ex vivo* studies). (*A*) Arteries isolated from FK506-treated (◇) and control (●) animals ($N = 9$ per group) were contracted with NE and challenged with a cumulative dose of Ach in basal conditions (asterisks indicate a significant difference between the two groups). Relaxation to Ach was also conducted after incubation with indomethacin or with a mixture of indomethacin and methylene blue. The maximum responses to Ach (expressed as percentage of precontraction with NE) in all three conditions are shown in (*B*). Symbols are: (■) control; (□) FK506-treated animals.

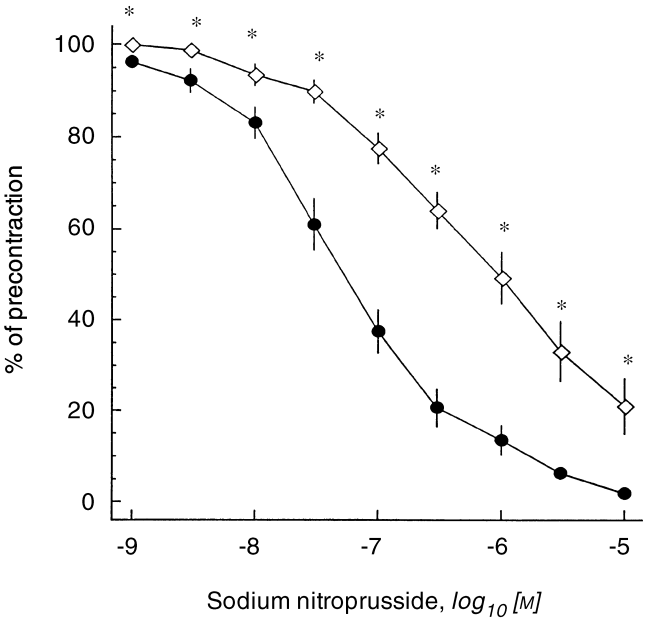

Fig. 8. Effect of FK506 (long-term treatment) on SNp-induced relaxation in isolated rat arteries (*ex vivo* studies). Arteries isolated from FK506-treated (◇) and control (●) animals ($N = 9$ per group) were contracted with NE and challenged with cumulative dose of SNp. Asterisks indicate a significant ($P < 0.05$) difference between the two experimental groups.

associated with a sustained and significant increase in blood pressure [13, 22].

FK506 did not change plasma creatinine or GFR (short-term study). This is in agreement with investigations showing the lack of acute effect of FK506 on GFR, renal vascular resistance, and renal plasma flow in the rat [23, 24]. On the other hand, a mild reduction in renal function in the animals exposed 21 days to the drug cannot be excluded because GFR was not measured in these experiments. Studies by others have shown that chronic administration of FK506 to rats under a low-salt diet [17] and to mice under an unrestricted diet [25] caused a reduction in renal function. If the same alteration occurred in our long-term experiments, it could be a plausible explanation for the sodium retention observed in the FK506 group. The most interesting aspect of our study is related to the functional observations in the resistance vessels, as they help to explain the reduced hypertensive properties of FK506 when compared with cyclosporine. In the short-term animal experiments, we found that resistance vessels of rats exposed to FK506 presented a reduced sensitivity to NE, preservation of the endothelium-dependent relaxation, and compromised endothelium-independent relaxation. A likely explanation for the reduced response to NE is that stimulation of sympathetic nervous system over one week or more could have induced the desensitization to the agonist. In this regard, Lyson et al showed that FK506 activates renal sympathetic nerves, a characteristic also shared with cyclosporine [19]. The fact that both drugs reduced NE sensitivity [13] reinforces this argument. The preservation of the endothelium-dependent relaxation (response to Ach) in the short-term experiments is, however, remarkable because it establishes a clear contrast with previous data demonstrating the adverse effect of cyclosporine on endothelial function [13, 26–28] and suggests that FK506 does not impair NO synthesis or release. Interestingly, the incubation of the resistance vessels with L-arginine, the NO synthase substrate, caused a significant reduction in Ach sensitivity in the rats treated with FK506 but not in controls. This suggests that the FK506 vessels might have produced more NO by comparison with controls during the incubation with L-arginine and become desensitized to the endothelial NO

released upon Ach addition. Saturation of vascular smooth muscle cell with excess NO may have also caused the reduction in SNp sensitivity observed in all our experiments and may have interfered with NE responsiveness, as reported previously [29–31]. It is tempting to speculate, at this point, that FK506 exerts a proinflammatory stimulus on the arteries and stimulates the expression of inducible NO synthase. Future experiments measuring both the expression and the activity of this enzyme in arteries or cultured vascular smooth muscle cells will be necessary to confirm this hypothesis.

Our short-term study on blood pressure responsiveness supports in part the conclusions of our vascular reactivity experiments discussed earlier here. First, rats treated with FK506 presented significant tachycardia at the baseline period compared with controls, despite similar levels of MAP. This strongly suggests that blood pressure was maintained by reflex increase in cardiac output in response to systemic vasodilation. Also, the rats receiving FK506 presented a reduced pressor responsiveness to exogenous NE, suggesting a possible activation of the sympathetic nervous system with secondary desensitization of adrenergic receptors as postulated in our *ex vivo* experiments. Experiments assessing sympathetic nervous system activity by the measurement of arterial NE levels, nerve traffic, or receptor expression will have to be undertaken to give further support to this hypothesis.

The activation of the sympathetic nervous system would also explain impaired MAP reactivity to the lowest doses of Ach employed (Fig. 6A). Surprisingly, and in apparent contrast with our *ex vivo* findings, higher doses of Ach in FK506-treated animals caused a significantly larger fall in MAP in comparison with the controls, and this effect was associated with a blunted heart rate response to the fall in blood pressure. We interpret these data as another manifestation of FK506-induced peripheral vasodilation. Thus, in our animals, similar to what is observed in hemorrhagic [30] or septic [32] shock, blood pressure would tend to fall and is barely maintained within normal range by sympathetic nervous system activation. Systemic challenge with high doses of Ach might have overcome this regulation and revealed the underlying vasodilation. *In vivo* experiments with selective adrenergic antagonists or NO synthase inhibitors may be useful in the future to confirm this hypothesis.

After 21 days, FK506-treated rats showed more marked alterations in vascular function compared with short-term studies. By the end of this period, we observed a modest but significant reduction in the maximum relaxation to Ach, which was not present in the short-term experiments. In addition, the vasodilatory response to SNp was further compromised because the maximum response was also impaired. Taken together, the data indicate that a more prolonged administration of FK506 may produce pathologic alterations in the endothelium, the smooth muscle cell, or both. The fact that the maximum relaxation could not be achieved either with Ach or SNp, however, points to a predominant defect in the smooth muscle cell rather than in the endothelium. Moreover, the failure for MB exposure to influence the response to Ach also suggests a more significant impairment of the smooth muscle. Finally, the reduction in the maximum relaxation apparently was not related to stimulation of endothelial thromboxane synthesis by Ach, as it was not reversed by indomethacin. In this regard, the absence of stimulatory effect of FK506 on the thromboxane pathway further contrasts with the effect of cyclosporine A, as noted by Benigni et al in their experiments with bovine aortic endothelial cells in culture [24].

The mechanism of smooth muscle injury secondary to FK506 treatment can only be speculated at this time. It is tempting though to propose that impaired vascular reactivity is a manifestation of drug-induced inflammation of the arterial wall and that excess inflammatory NO produced in response to the drug is the primary cause of cellular damage as postulated for certain glomerular diseases [33]. Interestingly, magnesium deficiency itself is capable of stimulating the production of inflammatory NO [34]. In this context, enhanced NO production following FK506 treatment could be the consequence of a direct activation of inducible NO synthase by the drug or be secondary to drug-induced magnesium depletion (Tables 1 and 2). Further investigations will be necessary to test each of these hypotheses.

In conclusion, we provide experimental evidence of the progressive nature of the vascular functional alterations associated with the use of FK506. These alterations differ from those observed with cyclosporine in being less pronounced and affecting predominantly the smooth muscle. The vascular changes are not associated with hypertension, but rather, with hemodynamic changes consistent with excess peripheral vasodilation. The alteration of both endothelium-dependent and -independent relaxation observed after long-term exposure to FK506 suggests, however, that the vascular manifestations of the drug toxicity may represent the forerunner of hypertension and other alterations seen with more prolonged use of the drug in the transplant population.

ACKNOWLEDGMENTS

Dr. Jose J.G. De Lima is a recipient of a grant from the Conselho Nacional de Pesquisa, Brasil. We thank Mr. Tongchan Phanouvong (Nephrology Division, Oregon Health Sciences University) for technical assistance and Ms. Molly Reusser (Nephrology Division, Oregon Health Sciences University) for careful review of the manuscript.

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